

WHAT IS CLAIMED IS:

5 1. An expression vector comprising:  
(a) a coding region with nucleic acids coding for a peptide product coupled in reading frame 3' of nucleic acids coding for a signal peptide; and  
(b) a control region linked operably with the coding region, said control region comprising a plurality of promoters and at least one ribosome binding site, wherein at least one of said promoters is tac.

2. The vector of claim 1, comprising a plurality of transcription cassettes, each cassette having said control region and said coding region.

3. The vector of claim 1, further comprising nucleic acids coding for a repressor peptide capable of repressing expression controlled by at least one of said promoters.

4. The vector of claim 3, wherein the nucleic acids coding for the repressor encode a lac repressor.

2 5. The vector of claim 1, wherein said control region has exactly two promoters.

3 6. The vector of claim 1, wherein said tac promoter is 5' of another promoter in said control region.

4 7. The vector of claim 1, wherein the control region comprises both a tac promoter and a lac promoter.

5 8. The vector of claim 4, wherein the lac promoter is 3' of the tac promoter.

6 9. The vector of claim 1, wherein said nucleic acids coding for the signal peptide encode a signal peptide for secreted bacterial proteins.

7 10. The vector of claim 4, wherein said signal is OmpA signal peptide.

8 11. The vector of claim 1, wherein said peptide product has a molecular weight of less than 10 KDa.

9 12. The vector of claim 1, wherein the C-terminal amino acid of said peptide product is glycine.

10 13. The vector of claim 9, wherein said peptide product is salmon calcitonin precursor.

11 14. The vector of claim 9, wherein said peptide product is calcitonin gene related peptide precursor.

15. The vector of claim 12, wherein said peptide product is selected from the group consisting of parathyroid hormone, the first 34 amino acids of parathyroid hormone, and a 35 amino acid peptide having a C-terminal glycine in position 35 and the first 34 amino acids of parathyroid hormone in positions 1-34.

16. The vector of claim 1, further comprising nucleic acids coding for at least one secretion enhancing peptide.

17. The vector of claim 16, wherein the secretion enhancing peptide is selected from the group consisting of secY and prlA-4.

18. The vector of claim 16, wherein the secretion enhancing peptide is secE.

19. The vector of claim 16, wherein a plurality of secretion enhancing peptides are encoded, at least one of which is secE and the other of which is selected from the group consisting of secY and prlA-4.

<sup>13</sup>  
~~20~~. A host cell transformed or transfected with the vector of claim 1.

21. A host cell transformed or transfected with the vector of claim 16.

<sup>14</sup> ~~22~~. The host cell of claim <sup>13</sup>~~20~~, wherein said host cell is a bacterial cell.

<sup>15</sup> ~~23~~. The host cell of claim <sup>14</sup>~~22~~, wherein said bacterial cell is a gram negative bacterial cell.

<sup>16</sup> ~~24~~. The host cell of claim <sup>14</sup>~~22~~, wherein said bacterial cell is *E. coli*.

25. The host cell of claim 24, wherein said *E. coli* is strain BLR.

26. The host cell of claim 24, wherein said *E. coli* is strain BL21.

<sup>19</sup>  
~~27.~~ The host cell of claim <sup>16</sup>~~24~~, wherein said *E. coli* is strain WA837.

28. The host cell of claim 22, wherein said host cell further expresses at least one secretion-enhancing peptide.

29. The host cell of claim 28, wherein the secretion enhancing peptide is selected from the group consisting of secY and prlA-4.

30. The host cell of claim 28, wherein the secretion enhancing peptide is secE.

31. The host cell of claim 28, wherein a plurality of secretion enhancing peptides are encoded, at least one of which is secE and the other of which is selected from the group consisting of secY and prlA-4.

<sup>20</sup>  
~~32.~~ An *E. coli* host containing and expressing an expression vector which comprises a plurality of transcription cassettes in tandem, each cassette comprising:

5 (a) a coding region comprising nucleic acids coding for a peptide product coupled in reading frame 3' of nucleic acids coding for a signal peptide; and

10 (b) a control region linked operably with the coding region, said control region comprising a plurality of promoters in tandem and at least one ribosome binding

site, wherein at least one of said promoters is selected from the group consisting of tac and lac.

21<sup>20</sup>~~23~~. The host of claim ~~22~~<sup>20</sup>, wherein said control region comprises from 5' to 3' a tac promoter and a lac promoter.

22<sup>20</sup>~~34~~. The host of claim ~~22~~<sup>20</sup>, wherein said vector has exactly two transcription cassettes in tandem.

23<sup>23</sup>~~25~~. A host cell transformed with an expression vector which comprises a gene for expressing salmon calcitonin precursor, said host cell being *E. coli* strain BLR.

24<sup>24</sup>~~38~~. A host cell transformed with an expression vector which comprises a gene for expressing calcitonin gene related peptide precursor, said host being *E. coli* strain BLR.

25<sup>25</sup>~~37~~. A method of producing a peptide product which comprises culturing the host cell of claim ~~26~~<sup>23</sup> in a culture medium and then recovering the peptide product from the medium in which the host cell has been cultured.

38. The method of claim 37 wherein peptide product yield exceeds 100 mg per liter of media.

39. A method of producing a peptide product which comprises culturing the host cell of claim 21 in a culture medium and then recovering the peptide product from the medium in which the host cell has been cultured.

40. A method of producing a peptide product which comprises culturing the host cell of claim 35 in a culture medium and then recovering the peptide product from the medium in which the host cell has been cultured.

29 41. The method of claim 25, wherein the peptide product is salmon calcitonin precursor.

29 42. The method of claim 25, wherein the peptide product is calcitonin gene related peptide precursor.

43. The vector of claim 1, wherein said peptide product is selected from the group consisting of parathyroid hormone, the first 34 amino acids of parathyroid hormone and 35 amino acid peptide having a C-terminal glycine in position 35 and the first 34 amino acids of parathyroid hormone in positions 1-34.

44. The method of claim 38, wherein a method of induction is started prior to stationary phase.

45. The method of claim 44, wherein the induction method is by addition of a chemical inducer.

46. The method of claim 45, wherein the induction is by addition of at least one inducer selected from the group consisting of IPTG and lactose.

47. A method of producing a peptide product which comprises the steps of:

(a) culturing host cells of claim 20 in a culture medium in the presence of a carbon source and

inducing the expression of the peptide product, while controlling growth of said host cells at a growth rate between 0.05 and 0.20 doublings per hour; and

(b) thereafter recovering said peptide product from the medium.

48. The method of claim 47, wherein a membrane-permeabilizing amount of glycine is present in the medium during at least a portion of said controlled growth.

49. The method of claim 47, wherein an inducer and carbon source are added during each hour of culturing in amounts such that the weight ratio of the inducer to the carbon source added in any one hour does not vary by more than 50% from the ratio added during the entire fermentation period.

50. The method of claim 47, wherein no external carbon source is introduced into the medium until carbon source initially present in said medium is depleted to a level at which it could not continue to support the life of said host absent introduction of external carbon source into the medium, and wherein carbon source is thereafter added at a rate which maintains said growth rate between 0.05 and 0.20 doublings per hour.

51. The method of claim 47, wherein the host cell is cultured for a period between 20 and 32 hours post induction.

53. The method of claim 47, wherein the host cell is cultured at a temperature between 20 and 35°C.

55. The method of claim 47, wherein the host cell is cultured at a temperature between 29.5 and 30.5°C.

57. The method of claim 47, wherein the pH of the culture medium is between 6.78 and 6.85.

59. The method of claim 47, wherein oxygen saturation of the culture medium is at least 20%.

60. The method of claim 47, wherein oxygen saturation of the culture medium is at least 50%.

61. The method of claim 47, wherein the oxygen saturation of the culture medium is at least 80%.



62. The method of claim 46, wherein the average cell growth rate during the culture period is maintained between 0.10 and 0.15 doublings per hour.

63. The method of claim 55, wherein the growth rate is maintained at about 0.13 doublings per hour.

64. The method of claim 49, wherein the carbon source is glycerol.

65. The method of claim 37, wherein said peptide product is salmon calcitonin precursor.

66. The method of claim 37, wherein said peptide product is calcitonin gene related peptide precursor.

67. The method of claim 37, wherein said peptide product is selected from the group consisting of parathyroid hormone, the first 34 amino acids of parathyroid hormone and 35 amino acid peptide having a C-terminal glycine in position 35 and the first 34 amino acids of parathyroid hormone in positions 1-34.

68. The method of claim 47, wherein induction is achieved by use of a chemical inducer.

69. The method of claim 58, wherein the inducer is at least one agent selected from the group consisting of lactose and IPTG.

70. The method of claim 37, wherein recovering said peptide product comprises:

(a) separating host cells from the culture medium; and

(b) subjecting the medium to reverse-phase liquid chromatography and recovering fractions containing peptide product; and

(c) subjecting said fractions of step (b) to cation exchange chromatography, and

(d) thereafter recovering fractions containing peptide product.

71. The method of claim 39, wherein recovering said peptide product comprises:

(a) separating host cells from the culture medium; and

(b) subjecting the medium to reverse-phase liquid chromatography and recovering fractions containing peptide product; and

(c) subjecting said fractions of step (b) to cation exchange chromatography, and

(d) thereafter recovering fractions containing peptide product.

72. The method of claim 47, wherein recovering said peptide product comprises:

(a) separating host cells from the culture medium; and

(b) subjecting the medium to reverse-phase liquid chromatography and recovering fractions containing peptide product; and

(c) subjecting said fractions of step (b) to cation exchange chromatography, and

(d) thereafter recovering fractions containing peptide product.

73. The method of claim 37, wherein recovering said peptide product comprises:

(a) separating host cells from the culture medium; and

(b) subjecting the medium to cation exchange chromatography and recovering fractions containing said peptide product; and

(c) subjecting the recovered fraction of step (b) to reverse-phase liquid chromatography and recovering fractions containing peptide product;

(d) subjecting the recovered fractions of step (c) to cation exchange chromatography, and

(e) thereafter recovering fractions containing peptide product.

74. The method of claim 39, wherein recovering said peptide product comprises:

(a) separating host cells from the culture medium; and

(b) subjecting the medium to cation exchange chromatography and recovering fractions containing said peptide product; and

(c) subjecting the recovered fraction of step (b) to reverse-phase liquid chromatography and recovering fractions containing peptide product;

(d) subjecting the recovered fractions of step (c) to cation exchange chromatography, and

(e) thereafter recovering fractions containing peptide product.

75. The method of claim 47, wherein recovering said peptide product comprises:

(a) separating host cells from the culture medium; and

(b) subjecting the medium to cation exchange chromatography and recovering fractions containing said peptide product; and

(c) subjecting the recovered fraction of step (b) to reverse-phase liquid chromatography and recovering fractions containing peptide product; and

(d) subjecting the recovered fractions of step (c) to cation exchange chromatography, and

(e) thereafter recovering fractions containing peptide product.

76. The method of claim 70, wherein the peptide product has at least one cysteine in its molecular structure and wherein at least one sulfhydryl group of a cysteine of the peptide product is sulfonated during at least a portion of said method of recovering peptide product.

77. The method of claim 71, wherein the peptide product has at least one cysteine in its molecular structure and wherein at least one sulfhydryl group of a cysteine of the peptide product is sulfonated during at least a portion of said method of recovering peptide product.

78. The method of claim 72, wherein the peptide product has at least one cysteine in its molecular structure and wherein at least one sulfhydryl group of a cysteine of the peptide product is sulfonated during at least a portion of said method of recovering peptide product.

79. The method of claim 73, wherein the peptide product has at least one cysteine in its molecular structure and wherein at least one sulfhydryl group of a cysteine of the peptide product is sulfonated during at least a portion of said method of recovering peptide product.

80. The method of claim 74, wherein the peptide product has at least one cysteine in its molecular structure and wherein at least one sulfhydryl group of a cysteine of the peptide product is sulfonated during at least a portion of said method of recovering peptide product.

81. The method of claim 75, wherein the peptide product has at least one cysteine in its molecular structure and wherein at least one sulfhydryl group of a cysteine of the peptide product is sulfonated during at least a portion of said method of recovering peptide product.

82. The method of claim 72, further comprising altering the pH of the culture medium, immediately after terminating fermentation, to a level where proteolytic degradation of product is reduced.

83. The method of claim 82, wherein salmon calcitonin precursor is the peptide product and pH is adjusted to between 2.5 and 4.0.

84. The method of claim 83, wherein the pH is adjusted to between 3 and 3.5.

85. The method of claim 82, further comprising lowering the temperature of the culture medium to below 10°C after fermentation is terminated.

86. A method of producing an amidated peptide product comprising the steps of:

(a) culturing the host cell of claim 20 in a culture medium wherein the peptide product includes a C-terminal glycine;

(b) recovering said peptide product from said culture medium; and

(c) converting said peptide product to an amidated peptide by converting said C-terminal glycine to an amino group.

87. A method of producing an amidated peptide product comprising the steps of:

(a) culturing the host cell of claim 35 in a culture medium wherein the peptide product includes a C-terminal glycine;

(b) recovering said peptide product from said culture medium; and

(c) converting said peptide product to an amidated peptide by converting said C-terminal glycine to an amino group.

88. A method of producing an amidated peptide comprising:

(a) culturing host cells which express a peptide product having a C-terminal glycine together with an N-terminal signal peptide under conditions wherein growth of said host cells is controlled to stay within a range of 0.05 to 0.20 doublings per hour; wherein the

culture is induced during some of the period of said controlled growth;

(b) recovering said peptide product from the culture media; and

5 (c) converting said peptide product to an amidated peptide by converting said C-terminal glycine to an amino group.

89. The method of claim 83, wherein the host cell is cultured in a culture medium in the presence of an inducer, while maintaining an average cell growth rate during culturing between 0.05 and 0.20 doublings per hour.

90. The method of claim 84, wherein the host cell is cultured in a culture medium in the presence of an inducer, while maintaining an average cell growth rate during culturing between 0.05 and 0.20 doublings per hour.

91. The method of claim 86, wherein the peptide product is salmon calcitonin precursor or calcitonin gene related peptide precursor.

92. The method of claim 87, wherein the peptide product is salmon calcitonin precursor or calcitonin gene related peptide precursor.

93. The method of claim 86, wherein said conversion to amidated peptide is accomplished by:

5 (a) forming a reaction mixture by contacting said peptide product with oxygen and a reducing agent in the presence of peptidyl glycine  $\alpha$ -amidating

monooxygenase, or peptidyl glycine  $\alpha$ -hydroxylating monooxygenase;

(b) if peptidyl glycine  $\alpha$ -amidating monooxygenase is not used in step (a), and if the reaction mixture is not already basic, then increasing pH of the reaction mixture until it is basic; and

(c) recovering said amidated peptide from said reaction mixture.

94. The method of claim 87, wherein said conversion to amidated peptide is accomplished by:

(a) forming a reaction mixture by contacting said peptide product with oxygen and a reducing agent in the presence of peptidyl glycine  $\alpha$ -amidating monooxygenase, or peptidyl glycine  $\alpha$ -hydroxylating monooxygenase;

(b) if peptidyl glycine  $\alpha$ -amidating monooxygenase is not used in step (a), and if the reaction mixture is not already basic, then increasing pH of the reaction mixture until it is basic; and

(c) recovering said amidated peptide from said reaction mixture.

95. The method of claim 88, wherein said conversion to amidated peptide is accomplished by:

(a) forming a reaction mixture by contacting said peptide product with oxygen and a reducing agent in the presence of peptidyl glycine  $\alpha$ -amidating monooxygenase, or peptidyl glycine  $\alpha$ -hydroxylating monooxygenase;

(b) if peptidyl glycine  $\alpha$ -amidating monooxygenase is not used in step (a), and if the



(c) recovering said amidated peptide from said reaction mixture.

96. The method of claim 93, wherein recovering amidated peptide comprises at least one of the steps selected from the group consisting of cation exchange chromatography and reverse phase chromatography.

97. The method of claim 94, wherein recovering amidated peptide comprises at least one of the steps selected from the group consisting of cation exchange chromatography and reverse phase chromatography.

98. The method of claim 84, wherein recovering amidated peptide comprises at least one of the steps selected from the group consisting of cation exchange chromatography and reverse phase chromatography.

99. A method for direct expression of a peptide product into a culture medium comprising the steps of:

(a) culturing host cells which express said peptide product together with a signal peptide, in said medium, under conditions wherein growth of said host cells is controlled to stay within a range of 0.05 to 0.20 doublings per hour; wherein an inducer is present during some of said period of controlled growth; and

(b) recovering said peptide product from the culture medium.

100. The method of claim 99, wherein a membrane-permeabilizing amount of glycine is present in the medium during at least a portion of said period of controlled growth.

101. The method of claim 99 wherein oxygen saturation averages over 50% in said medium during the period of controlled growth.

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